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Poly-L/D-lactide stents as intravascular devices – an experimental study

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Academic dissertation

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CONTENTS

ABBREVIATIONS

ELISA	**Enzyme-linked immunosorbent assay**
F1+2	**Prothrombin fragments 1+2**
GFP	**Gel-filtered platelets**
MRA	**Magnetic resonance angiography**
MR/ MRI	**Magnetic resonance/magnetic resonance imaging**
NZW	**New Zealand White**
PBS	**Phosphate buffered saline**
PCL	**Polycaprolactone**
P(CL/D,L-LA)	**Copolymer of ε-caprolactone and D,L-lactide**
P(CL/L-LA)	**Copolymer of ε-caprolactone and L-lactide**
PGA	**Polyglycolic acid**
PLA	**Polylactide**
PPACK	**D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone**
PPP	**Platelet-poor plasma**
PRP	**Platelet-rich plasma**
PTA	**Percutaneous transluminal angioplasty**
PVC	**Polyvinylchloride**
SD	**Standard deviation**
SEM	**Scanning electron microscopy**
SS	**Stainless steel**
TAT	**Thrombin-antithrombin complex**
TT	**Thrombin time**

1. LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to hereafter by their Roman numerals:

I Hietala E-M, Salminen U-S, Ståhls A, Välimaa T, Maasilta P, Törmälä P, Nieminen MS, Harjula ALJ: Biodegradation of the copolymeric polylactide stent. Long-term follow-up in a rabbit aorta model. J Vasc Res 2001;38:361-369.

II Hietala E-M, Maasilta P, Ståhls A, Salminen U-S, Harjula ALJ, Välimaa T, Kivisaari L: Magnetic resonance evaluation of luminal patency after polylactide stent implantation: an experimental study in a rabbit aorta model. Eur Radiol 2003;3:1025-1032.

III Hietala E-M, Maasilta P, Välimaa T, Harjula ALJ, Törmälä P, Salminen U-S, Lassila R: Platelet responses and coagulation activation on polylactide and heparin-polycaprolactone-L-lactide -coated polylactide stent struts. J Biomed Mater Res 2003;67A:785-791.

IV Hietala E-M, Maasilta P, Juuti H, Nuutinen JP, Harjula ALJ, Salminen U-S, Lassila R: Platelet deposition on stainless steel, spiral and braided polylactide stents: a comparative study. Thrombosis and Haemostasis (submitted).

2. ABSTRACT

Therapeutic balloon angioplasty causes an abrupt injury to a vessel wall and alters elements of vascular wall repair, especially inflammatory processes. The net benefit of stent implantation is an attribute to the larger initial lumen gain and the prevention of negative remodelling. In addition to in-stent restenosis, stent implantation may be complicated by (sub)acute thrombosis, or by possible rare complications, such as infection, vessel perforation, immunologic reaction, or corrosion. As a result, various antithrombotic and antiproliferative stent coatings and alternative stent materials have been developed. The aim of this study was firstly to evaluate the utility of polylactide (PLA) as stent core material using simple stent prototypes. Secondly, thrombogenicity of PLA in comparison with stainless steel was assessed. An additional goal of this study was to explore whether it was feasible to improve the haemocompatibility of PLA by coating it with unfractionated releasable heparin.

Twenty adult New Zealand White (NZW) rabbits were used as test animals for surgical aortic PLA stent implantation. Tissue reactions were evaluated at various time points from one to 34 months after stent implantation by histologic assessments and by scanning electron microscopy (SEM). In general, tissue reactions were mild in response to stent implantation, and the PLA stents degraded within 24 months. The conclusion was that PLA is useful as a stent core material for future biodegradable stent designs.

Next, the possibility of measuring intraluminal dimensions by magnetic resonance angiography (MRA) after PLA stent implantation was investigated. Twelve spiral PLA stents and six stainless steel (SS) stents were implanted into NZW rabbit aortas, with additional six rabbits serving as a non-operated control group for imaging studies. Aortic measurements were established with a 1.5 T magnetic resonance (MR) scanner by the use of computerised measurements. Six PLA-stented

animals were followed-up to one year after stent implantation, and histology and SEM were performed at the end of the study. PLA stents were translucent in conventional MR scanning, while SS caused a signal loss. In conclusion, PLA stents allow luminal visualisation by MRA straight after stent implantation and during the early phase of biodegradation. However, a marking system should be created for small-vessel PLA stents.

Signs of blood compatibility of stainless steel and unheparinised and heparinised polylactide stent struts were assessed by using a steady state model with human blood. The aim was firstly to challenge the natural anticoagulative factors, and secondly, to assess the procoagulant capacity of platelets in the presence of a stent strut. Platelet deposition and morphology were studied, as well as various markers for coagulation activation. At physiological platelet densities, more platelets adhered to SS than to PLA per stent strut area. Thrombin time (TT) values were prolonged in all assessments for heparin-coated PLA struts. The interpretation was that PLA is at least as haemocompatible as stainless steel, and when heparinised, PLA may be of further benefit.

Finally, blood compatibility of different PLA stents in comparison with stainless steel stents was examined applying a whole blood perfusion model. The stents were first perfused without precoating, and then, to mimic the pathophysiology of balloon injury related to stenting, by precoating the stent containing segment of the perfusion tubing with fibrillar type I collagen. Quantitively, SS stents collected the least platelets under all study conditions. Among all biodegradable stents, the braided stent coated with polycaprolactone-polylactide-heparin accumulated the fewest platelets. In conclusion, under flowing conditions, PLA stents were not as haemocompatible as SS stents, but heparin coating increased the blood compatibility of PLA stents. Therefore, the design of biodegradable stents must be further developed before clinical studies are planned.

3. INTRODUCTION

Percutaneous transluminal angioplasty (PTA), with or without stenting, has become a treatment of choice in selected patients with coronary and peripheral atherosclerotic lesions (Fischman et al.1994, Serruys et al. 1994, Hsieh et al. 2001, TASC Investigators 2000). Despite the indisputable advantages of PTA, this kind of treatment may be complicated by (sub)acute thrombotic occlusion or restenosis (Schömig et al.1996, Fischman et al.1994), or by possible stent-related complications, such as infection (Therasse et al. 1994, Hearn et al.1997, Doslouglu et al. 2001), vessel perforation (Smith et al. 2001, Korpas et al. 2002), immunologic or foreign-body reactions (Yutani et al. 1999), or corrosion (Ryhänen et al. 1997). Since the introduction of stents to clinical practice over 20 years ago, much effort has been expended to deal with thrombogenicity and in-stent restenosis. The development of periprocedual medications, and in particular introduction of antiplatelet and anticoagulative treatment in the modern era, have greatly diminished rates of early thrombotic occlusions (Orford et al. 2002). The problem of in-stent restenosis appears to be remarkably delayed, or perhaps even resolved, by the use of novel antiproliferative stent coatings such as sirolimus (rapamycin) and paclitaxel (Morice et al. 2002, Park et al. 2003, Schampaert et al. 2004).

Balloon angioplasty causes abrupt injury to the vessel. Adhesion, activation, aggregation, and deposition of platelets as well as inflammatory cells occur at the injured site. After the thrombotic period of healing, the repair process takes a much longer time, at least several weeks, firstly in the recruitment phase and secondly in the proliferative phase (Hofma et al. 2001). Despite the positive effects of stents, especially prevention of elastic recoil and abrupt vessel closure, these conventional permanent devices can cause a longer-lasting injury to the vessel wall and more pronounced long-term endothelial dysfunction than simple PTA (van Beusekom et al. 1998). Implanting a stent is introducing a foreign body into tissue and into contact with blood, thus biocompatibility and

haemocompatibility are two major issues that must be dealt with. Consequently, it seems logical that prevention of these possible undesirable effects by improving stent properties would be more effective than treatment of the complications.

Therefore, both different stent coatings and alternative stent materials have been developed. Heparin- and hirudin-coatings have been directed against the clotting cascade (Seifert et al. 1995, Alt et al. 2000). Various antiproliferative agents, such as angiopeptin analogues, methotrexate, dexamethasone, paclitaxel, and sirolimus among others (Cox et al. 1992, De Scheerder et al.1996a, Lincoff et al.1997, Betrtrand et al. 1998, Morice et al. 2002, Park et al 2003), as well as irradiation (Tierstein and Kuntz 2001) have been used to fight against the proliferative process leading to intimal hyperplasia and restenosis/in-stent restenosis. On the other hand, corrosion of metals may stimulate fibroblast growth, platelet deposition, and protein adhesion, which consequently may cause stenosis in the stented area (Ryhänen et al.1997, Shih 2001), but this has not yet been proven with stents. Furthermore, Hoffmann et al. (1996) have suggested that some early positive effects of stent implantation may be diminished by hindrance of the late, favourable luminal remodelling. As a result, new stent materials, either biostable or biodegradable, have been sought.

Among biodegradable materials, polylactide (PLA) is one of the most employed materials in medicine, showing excellent biocompatibility in various tissues, but not without controversy in vascular applications, mainly due to the previously described inflammatory reactions (van der Giessen et al. 1996, Lincoff et al.1997**,** Su et al. 2003). Currently available stents are made of various metals and are mostly permanent. Theoretically, bioabsorbable devices could offer some benefits over permanent ones. They would not remain as foreign bodies in the target tissue, after serving as intravascular dilators through the most vulnerable phases of healing after PTA, allowing positive late luminal remodelling. Therefore, any recurrent procedure, in the form of repeated PTA

or revascularisation, would not be interfered with. Secondly, the absorption time in tissues could be tailored, as this depends on the choice of basic molecule(s), degree of polymerisation, internal arrangement of the material components, stent configuration, method of sterilisation, site of implantation (Törmälä et al. 1998), and biomechanical stresses and forces (Miller and Williams 1984). Thirdly, in addition to serving as small vessel dilators and as carriers for drugs and genes**,** bioabsorbable devices could be used in the treatment of venous or congenital vascular strictures or peripheral arteriosclerotic lesions, extending the applications of stents (Bertrand et al. 1998).

This study was designed firstly to examine tissue compatibility of poly-L/D-lactide stents in small vessels in a normal rabbit aorta model, and secondly, to experimentally evaluate signs of early biodegradation and determine the possibility for measurement of luminal dimensions after stent implantation by magnetic resonance angiography (MRA). Finally, thrombogenicity of polylactide in comparison with stainless steel was investigated by two different *in vitro* models with human blood. The effect of coating the core material, PLA, with heparinised as well as non-heparinised resorbable materials, was also assessed with these *in vitro* human blood models.

4. REVIEW OF THE LITERATURE

4.1. Treatment of atherosclerosis

Therapeutic regimens in atherosclerosis have undergone a fundamental change during the past few decades, not only due to better understanding of the cellular and molecular mechanisms involved in the development of the disease, but also as a result of the development of both medical treatment and interventional techniques. Furthermore, the "conservative treatment" is no more conservative, and instead consists of an active policy to decelerate progression of the disease and to prevent complications. This includes controlling risk factors such as high lipid levels, hypertension, smoking, lack of exercise, and obesity. Furthermore, preventive medical treatment of risk groups, especially of diabetic patients, includes therapy with β-blockers, statins, angiotensin converting enzyme (ACE) inhibitors, and antiplatelet/anticoagulative agents (Pearson et al. 2002, Caprie steering committee 1996, Bhatt and Topol 2000, Bhatt et al. 2002, Steinhubl et al. 2002, Serruys et al. 2002, Gomma et al. 2002). Ongoing and future trials will evaluate the role of antiplatelet regimens in the prevention and management of coronary, cerebral, and peripheral vascular diseases.

When it comes to interventional techniques, along with the introduction of advanced percutaneous techniques a revolution has taken place over the past 20 years. Conventional bypass surgery, in the treatment of both coronary and peripheral artery lesions, has quite often become "the last chance" for revascularisation. Instead, PTA, combined often with stenting and always with appropriate medical therapy, is the predominant therapeutic option in the treatment of chronic ischemia due to coronary or supragenicular atherosclerotic lesions, whenever lesion morphology and the clinical situation are favourable for this kind of procedure. Acute ischemic attacks can often be managed by either thrombolysis – frequently combined with concomitant percutaneous intervention – or bypass surgery, depending on the clinical situation and available medical and technical resources.

Endoluminal grafting is widely applied in clinical practice, although its position as a treatment option is under evaluation, as long-term results are being published (Harris et al. 2000, Aho et al. 2002, Bray et al. 2003). The possible benefit from minimally invasive surgical approaches, in both coronary and peripheral vascular surgery, is also under investigation (Acikel et al. 2001, Turnipseed et al. 2003, Ohtsuka et al. 2003, Bonatti et al. 2003). Combining percutaneous and surgical approaches, i.e., hybrid techniques have also become clinical practice in selected cases (Melliere et al. 1999, Sinci et al. 2000, Lawrence-Brown et al. 2000, Schneider et al. 2001, Stahl et al. 2003).

4.2. Evolution of percutaneous transluminal angioplasty (PTA) and stenting

Over 50 years ago, the Swedish doctor Sven-Ivar Seldinger introduced the idea of a percutaneous entry technique to gain access to any part of the cardiovascular system (Seldinger 1953). Roughly a decade later, Dotter and Judkins (1964) described the principle of transluminal catheter dilation, and the idea of endovascular tubular grafting was introduced in 1969 (Dotter 1969). The invention of a balloon dilation catheter created a real revolution in the treatment of atherosclerotic lesions, and the first clinical results of non-operative dilation of coronary arteries were reported in 1978 (Grüntzig 1978). Along with the development of new intravascular supporting devices, the term “stent” was introduced, based on the surname of the British dentist Charles Stent, who had first applied a related device in 1856 (Ring 2001). Currently, PTA, often combined with stenting, has become the first choice of interventional therapy in most coronary, renal, and suprainguinal peripheral artery lesions. In coronary arteries, stents markedly reduce restenosis rate (Kiemeneij et al. 2001, Schiele et al. 2003, Brophy et al. 2003), and they can be used to prevent abrupt vessel closure and to treat vessel dissections, thus reducing the need for acute operative interventions.

The treatment of infrainguinal atherosclerotic lesions by PTA remains challenging, as the long-term patency rates have been somewhat disappointing, despite good primary success. Often, such as in

the case of critical limb ischemia, selection between percutaneous, hybrid, or surgical approaches depends greatly on each individual's concomitant diseases and operability, in addition to the morphologic angiographic situation (TASC Investigators 2000). Presently, PTA is applied widely in the treatment of venous disorders, although long-term reports are scarce (Lamont et al. 2002, Raju et al. 2002, De Gregorio et al. 2003, Kalra et al. 2003). New antiplatelet drugs and the development of stent design and coatings, however, may both improve long-term results of stenting and extend applications of stents in the near future.

4.3. Vascular response to PTA and stenting

Nowadays, the differences between vascular biological responses to balloon- and stent-induced injuries are recognised (Schober et al. 2002). Welt and Rogers (2002) have presented an integrated view of the pathophysiologic events underlying in-stent restenosis (Figure 1). Deendothelialisation after stent implantation is followed by immediate deposition of a layer of platelets and fibrin at the injured site. Adhesion molecules (e.g. P-selectin and glycoprotein Ibα) secreted by activated platelets attach to circulating leukocytes via platelet receptors, and a process of rolling along the injury site begins (Kuijper et al. 1998). With the aid of cytokines, leukocytes bind to the leukocyte integrin class of adhesion molecules through direct attachment to platelet receptors, cross-linking fibrinogen to the glycoprotein IIb/IIIa receptor. Smooth muscle cells and resident leukocytes release cytokines, which stimulate the migration of leukocytes across the platelet-fibrin layer into the tissue. Platelets, leukocytes, and smooth muscle cells release growth factors, which influence the migration of smooth muscle cells from the media to the neointima and their proliferation. In this phase, the formed neointima has smooth muscle cells, extracellular matrix, and macrophages, recruited over a period of several weeks. In the final phase of healing, cellular elements are replaced by a production of extracellular matrix, and at least partial reendothelialisation takes place (Welt and Rogers 2002), usually within three weeks (Lindner et al.1993). Monocyte-derived

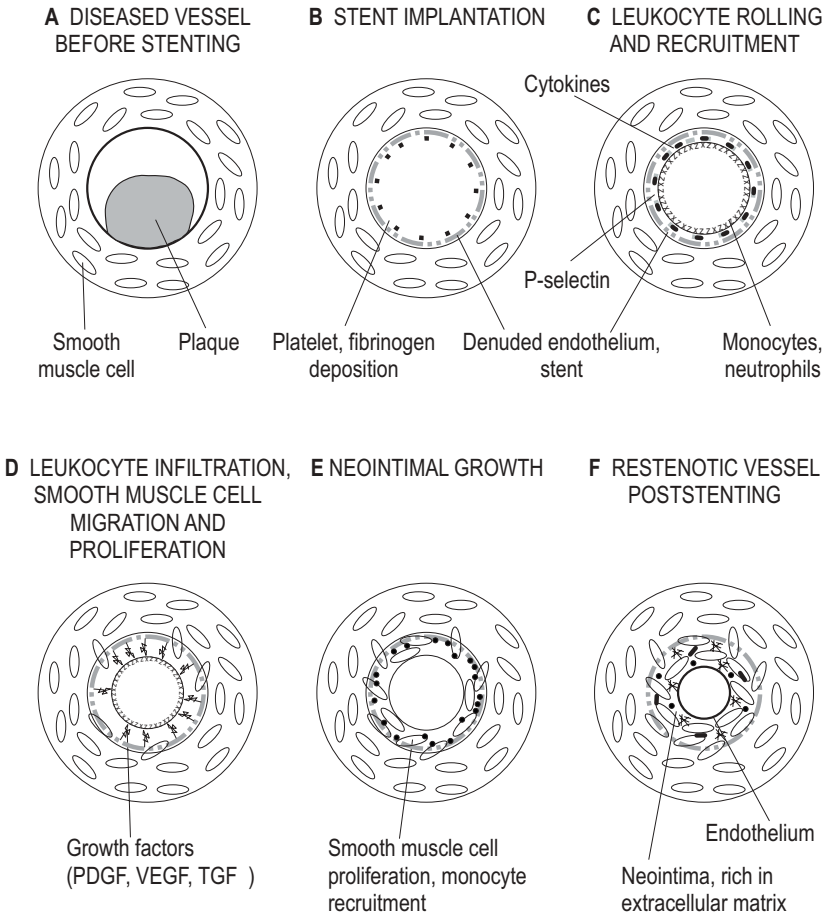

Figure 1. Cross-sectional, simplified presentation of pathophysiologic events in the intima and media after percutaneous angioplasty and stenting resulting in in-stent restenosis. Modified from Welt and Rogers, 2002. Stent implantation leads to endothelial denudation and deposition of platelets and fibrinogen (B). Leukocyte rolling, recruitment, and infiltration, and smooth muscle cell (SMC) migration and proliferation occur during the first days after vascular injury (C and D). Neointimal thickening is detectable within a few weeks following the injury, and SMC proliferation and monocyte recruitment continue (E). During the following weeks and months, the cellular content of the neointima diminishes and is replaced through production of extracellular matrix (ECM). Finally, a restenotic lesion develops. PDGF = Platelet-derived growth factor, TGFβ = Transforming growth factor-beta, VEGF = Vascular endothelial growth factor

macrophages seem to be associated with lesion progression, due to persistent secretion of cytokines and growth factors (Ip et al.1990). Some recent data suggest that somatic stem cells may also play a pivotal role in the pathological remodelling of the vessel wall after angioplasty (Sata et al. 2002, Kang et al. 2004).

Clinically, stenting appears to incur greater neointimal growth than PTA, but the net benefit of stent implantation is attributed to the larger initial lumen gain and the prevention of negative remodelling (Fischman et al. 1994, Serruys et al. 1994, Hoffmann et al. 1996). Furthermore, there is some evidence that an increased inflammatory response may explain the larger neointimal growth in stented arteries than in balloon-dilated arteries (Inue et al. 2000). In clinical stent thrombosis, the role of platelets is also known to be essential, coinciding with such factors as the extent of vascular injury, errors in stent design and engineering, incomplete stent expansion, ineffectiveness of antithrombotic therapy, and the thrombogenic nature of metallic surfaces. In addition to activated endothelial cells, platelets contribute to accumulation of leukocytes and to changes in their behavior in both physiological and pathological conditions, emphasising the intimate relationship between thrombosis and inflammation (Weyrich et al. 2002**)**.

4.4. Stent materials

Material characteristics, bulk and surface properties, stent configuration, and chemistry are pivotal factors to be taken into account in the creation of an ideal intravascular stent. An optimal stent should be easily delivered with appropriate expandability, strength, and elasticity. Furthermore, it should be haemocompatible, allowing complete endothelialisation and positive vessel remodelling, and it should induce neither neointimal overgrowth nor restenosis. To date, all clinically applied stents are made of various metals, although there have been numerous attempts to design stents made of other materials over the past decades. All stent materials can be roughly divided into two

main categories, biostable or biodegradable/absorbable, but as stent coatings have recently been applied both experimentally and clinically, a third category of partly biostable, partly degradable stents has emerged, into which category many coated stents fall.

4.4.1. Biostable materials

The first stents introduced were coilsprings made of stainless steel (SS) wire (Dotter 1969). The most applied stents are nowadays made of stainless steel, nitinol (nickel-titanium) or tantalum. SS is most often 316L alloy, L indicating low carbon content, with the predominant contents being iron, chromium, and nickel. The chromium contributes strength and hardness, and good corrosion protection (Park 1995). Self-expanding nitinol alloys have shown good biocompatibility as stents, although there is a theoretical concern of possible nickel leakage resulting in immunogenic reactions. Devices made of tantalum have a few theoretical advantages over SS: radiopacity, biocompatibility, mechanical properties, and lack of ferromagnetism. *In vivo* tantalum stent wires undergo oxidation, which results in extremely stable surface properties (Taylor 1996). Polishing appears to reduce thrombogenecity of SS and nitinol stents (De Scheerder et al. 1998, Sheth et al. 1996a, Fontaine 1996)**.** Clinically, however, it is uncertain if there is any advantage *in vivo* based on the choice of the basic metal itself. Stent design, surface and mechanical properties, and stent-induced vessel injury seem to be more important issues (Fontaine et al.1994, Hehrlein et al. 1995, Barth et al. 1996, Sheth et al. 1996a, De Scheerder at al. 1998, Taylor et al. 2001, Farb et al. 2002).

4.4.2. Biodegradable materials

Polylactide (PLA)

Since their introduction as bioabsorbable surgical materials in the 1960's and 1970's, polymers such as polyglycolic acid (PGA) and PLA have been widely used as suture material (Dexon®, Vicryl®), and as fixation devices (screws, pins, plates) in orthopaedics, traumatology, and plastic surgery

(Böstman et al. 1990, Törmälä and Pohjonen 1995, Suuronen et al.1999, Weisberger and Eppley 1997). As tubular duct devices, stents made of polymers have been introduced in urology (Petas et al. 1997), gastroenterology (Parviainen et al. 2000) and, in the future, perhaps as an alternative to treat airway stenosis as well (Korpela et al. 1999). Whether biodegradable materials are suitable in routine intravascular applications is still, however, under evaluation (Michalis 2002, Tamai 2000).

Polylactic acid is derived from the cyclic diester of lactic acid by ring-opening polymerisation, producing a poly-α-hydroxy derivate of the original acid (Gilding and Reed 1979). The clinically important polymers of PLA are macromolecules with molecular weights from tens of thousands of daltons to more than one millon daltons. The asymmetric polylactic acid has two st ereoisomeric forms, L- and D-isomers (Cutright et al. 1974, Connor et al. 1983) (Figure 2). The L-isomer exists in normal human carbohydrate metabolism, and the D-isomer is detectable in urine and in acidic milk. If a polymer is formed by one type of monomer, it is called homopolymer (e.g. poly-L-lactic acid, PLLA). A copolymer consists of two types of monomers (e.g. poly-D,L-lactic acid, PDLLA). The strength of the polymer depends on the basic molecule, the length of the chain, and the microstructure (Törmälä 1992). In general, randomly oriented and loosely -packed polymeric chains are amorphous and weak. On the contrary, parallel, tightly-packed polymers are more crystalline and stronger. Such polymers are self-reinforced through the die-drawing method, in which long molecules become parallel and form microfibrils increasing strength, modulus, and toughness (Törmälä 1992). As part of the microstructure is oriented into reinforcement elements, the material receives metal-like properties.

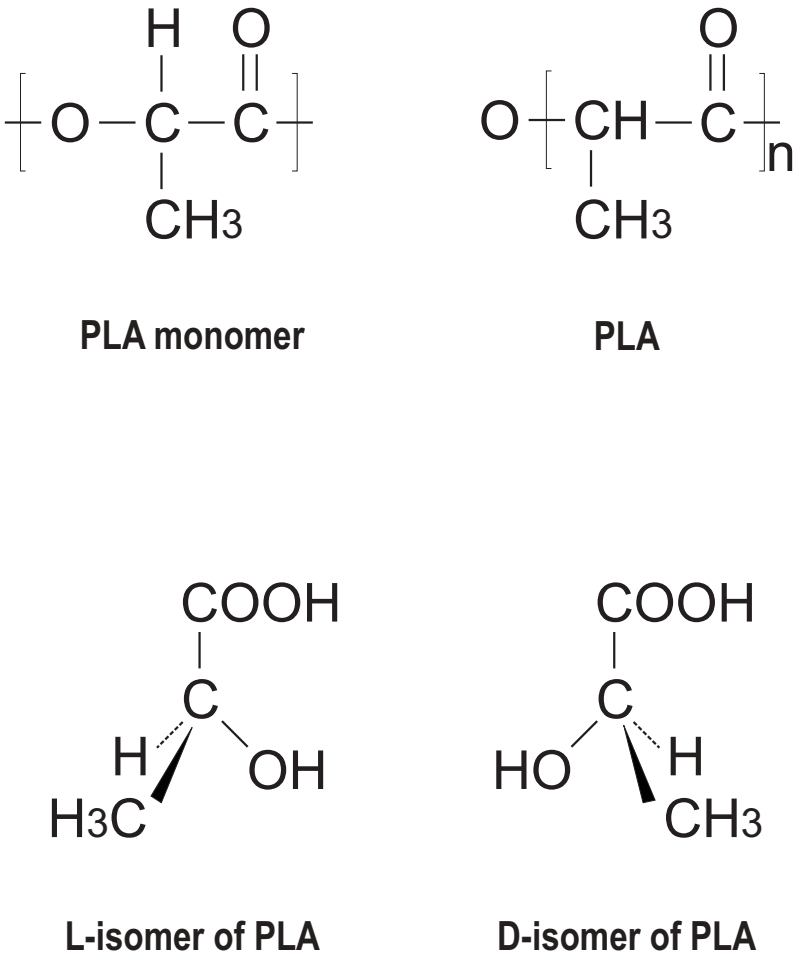

Figure 2. Polylactide is polymerised from a lactic acid dimer. Lactic acid occurs in two stereoisomeric forms, L(+) and D(-).

Biodegradation of a polymer implanted in a biological environment can be defined as its disintegration by hydrolysis, enzymes, or bacteria (Chu 1981). The speed of biodegradation depends on the choice of the basic molecule(s), molecular weight, configuration and thickness of the material, site of implantation, biomechanical stress to which the implant is exposed, and

impurities (presence of low molecular weight compounds, monomers, and D-isomers) (Törmälä 1992, Törmälä et al. 1998, Miller and Williams 1984). Poly-L-lactide is de-esterified into lactic acid by hydrolysis and converted to puruvate and then to acetyl-CoA in the citric acid cycle in the mitochondria. The end products carbon dioxide and water are excreted via respiration and urine (Kulkarni et al. 1966, Brandy et al. 1973, Williams 1982, Hollinger and Battistone 1986). The metabolism of poly-D-lactide is presumably enabled by a mitochondrial enzyme, which catalyses the oxidation of D-lactate to pyruvate (Connor et al.1983). The degradation of PLA can be accelerated by cellular enzymes (Williams 1982, Vasenius et al. 1990) and free radicals (Williams 1992, Ali et al. 1993).

The *biocompatibility* of PLA is relatively good in biological environments other than intravascular, as evidenced by limited, mild inflammatory reactions and foreign body reaction around the implanted material (Kulkarni et al. 1966, Cutright et al. 1971, Cutright and Hunsuck 1971, Rokkanen et al. 1985, Majola et al. 1991, Törmälä 1992). Rarely, an aseptic soft tissue response after bone plate fixation is possible, presumably due to remnants of slowly degrading PLA (Bergsma et al. 1995). Stents made of PLA, as well as PGA, have previously been employed with promising results, as a treatment of choice after prostatectomy to avoid voiding problems (Talja et al. 1995, Petas et al. 1997). In addition, experimental data have demonstrated excellent tissue compatibility in the treatment of experimental tracheal stenosis (Korpela et al. 1998).

The experimental data of PLA's biocompatibility in intravascular applications has been partly controversial so far: the inflammatory responses have varied from very mild to severe. The first *in vivo* studies with a bioabsorbable stent were performed by researchers at Duke University in the late 1980's (Zidar 1994). This stent was made of poly-L-lactide, with at least comparable or even better radial strength than a comparable stainless steel stent. The stents, with a maximal lumen of 4

mm, were dilated in femoral arteries of 11 mongrel dogs, and followed up to two years after implantation. Of the 11 stents, nine remained patent for follow-up. Histologic sampling revealed minimal thrombus formation, moderate neointimal growth, and limited inflammatory reaction. The stent had disintegrated by 9 months. Some data indicates that an inflammatory reaction depends greatly on the molecular weight of the implanted polymer. In metallic stents coated with low molecular weight (80 kDa) poly-L-lactide, Lincoff et al. (1997) noted more severe vascular response with acute and chronic inflammation than in those coated with high molecular weight (321 kDa) poly-L-lactide. The only human study with biodegradable poly-L-lactide stents was published recently (Tamai et al. 2000), and had a follow-up period of six months after the stent implantation in coronary arteries. The long-term data, including the presumable period of stent disintegration, have not been reported.

Polycaprolactone (PCL)

Poly-epsilon-caprolactone (PCL) is, like PLA, a biodegradable aliphatic polyester which undergoes a two-stage degradation process: firstly, hydrolytic cleavage of ester groups begins non-enzymatically, and then in the second phase the polymer becomes more highly crystalline, and its molecular weight is reduced. Experimental studies suggest that the intracellular degradation of PCL is the main pathway of degradation *in vivo* (Woodward 1985). When equal quantities of L-lactide and epsilon-caprolactone are synthesised to form a copolymer, crystallised PLA blocks seem to be much more resistant to biodegradation than PCL *in vitro* (Li et al. 1996). PCL has been tested as a carrier substance in various kinds of tissue engineering experiments, for drug or gene transfer, often as copolymeric material (Kweon et al. 2003, Woo et al. 2000, Ye et al. 1998). Clinically, a triblock-copolymer of PCL and polydimethylsiloxane has been tested in cardiopulmonary bypass surgery as an antithrombogenic circuit coating (Rubens et al. 1999).

Perhaps one of the most referred-to experiments ever regarding biocompatibility of biodegradable materials, in which PCL was included, was published by van der Giessen et al. (1996). Tantalum wire stents were covered with a 90 degree arc of non-sterile polymer and then deployed in porcine coronary arteries. The polymers were copolymers of PGA/PLA (PGLA), polycaprolactone (PCL), and of polyhydroxybuturate/hydroxyl-valerate (PHBV). In all procedures, the stented arteries remained patent to the end of the follow-up of 28 days, but some stenosis was noted due to severe inflammation and cell infiltration, with giant cells, leukocytes, lymphocytes, and mononuclear and eosinophilic cells detectable at the polymeric site in all cases. No bacteria were found at Gram staining. The molecular weights of the polymers were not reported. The authors concluded that these intense reactions were caused by the implanted polymers. It is possible, however, that differences in molecular weight have a strong effect on tissue response (Gogolewski et al.1993, Lincoff et al. 1997). No other studies have suggested such intense reactions related to the degradation process of either PCL or PLA.

Other biodegradable stent materials

Bier et al. (1991) reported the first attempt to use type I collagen as a stent material. This was a tubular, self-expanding stent with a negative charge to increase its haemocompatibility, tested *in vitro*. Subsequently, stents made of polyglycolic acid were examined by a Japanese group in the early 1990's (Susawa et al. 1993). These stents were implanted into canine coronary arteries, and followed up to 8 weeks. The degradation of this polymer was very rapid, thrombus formation remarkable, and the radial force decreased already after one week. Gao et al. (1996) have developed a copolymeric PLA-PCL stent impregnated with heparin; these stents were tested for two months in a porcine model with favourable results. Most recently, bioabsorbable stents made of of magnesium (>90%) have been developed. Heublein et al. (2003) tested such an alloy in a porcine model with favourable results. An encouraging study with bioabsorbable magnesium

stents in human arteries has also been reported (Marc Bosiers, oral presentation, Charing Cross International Symposium, London, 3th-6th April, 2004). These stents were percutaneously delivered after angioplasty into human superficial femoral arteries in a small series of patients with promising short-term results.

4.4.3. Coated stents

Stent coatings have been developed for two main purposes: for ameliorating haemocompatibility and for diminishing neointimal overgrowth. Several techniques (dipping, spraying, plating, sputtering, and surface-induced mineralisation) as well as coatings (inorganic, ceramic, and polymeric materials, immobilised or releasable drugs with degradable or stable matrices) have been employed. Some of these materials have been tested for effective coating, and others have been used for efficient drug delivery (Bertrand et al.1998). In addition, endothelial cell seeding, as well as surface-incorporated gene vectors have been tried on stents (Ye et al. 1998, Yamawaki et al. 1998, Scott et al. 1995, Kutryk et al. 1998, Flugelman et al. 1992, Dichek et al. 1989). Despite encouraging experimental data, many of these coatings have failed clinically, and even adverse effects have occurred (Kastrati et al. 2000a).

Antithrombotic stent coatings

Among thromboresistant stent coatings, heparins are the most extensively investigated drugs. They can be absorbed or immobilised on a stent surface in three ways: ionically, by mixing with a polymer, or by covalent binding (Table 1). The principal anticoagulant mechanism of unfractionated heparin is dependent on its interaction with antithrombin III, which results in inactivation of thrombin and other coagulation factors (Lindahl et al. 1979, Thunberg et al. 1982). A specific carbohydrate sequence (pentasaccharide) of the heparin molecule is essential for this, and must thus be preserved through the stent coating process. First tested as a coating in

cardiopulmonary circuits with favourable results, Duraflo II® coating applies ionic bonding, in which heparin is releasable from the surface to some extent (De Scheerder et al. 1997, Matsumoto et al. 2002). Matsumoto et al. used releasable heparin, and found that the antithrombin activity of heparin was dose-dependent. Gao et al. (1996) applied copolymeric PCL/PLA stents impregnated with heparin in an experimental model with encouraging results. However, a problem with these first two heparin-bonding methods may be the uneven or unpredictable content and activity of bound heparin.

TABLE 1. PRINCIPLES OF HEPARIN BONDING

	MECHANISM	USE
IONIC BONDING	Heparin activity is retained on the surface. Heparin release occurs to some extent.	Extracorporeal circuits Stent coatings
POLYMER MIXTURE	Heparin is released according to (co)polymeric degradation.	Experimental stent coatings
COVALENT BONDING	The anticoagulative activity of heparin depends on preservation of free antithrombin binding sites of the molecule. Heparin in not released from the surface.	Stent coatings Coatings in bypass grafts

Classic covalent bonding of heparin is applied in Carmeda® coatings (Larm et al. 1983, Hårdhammar et al.1996, Serruys et al. 1996). With this kind of coating, approximately 15% of the end-point-attached heparin molecules preserve the high affinity to bind antithrombin III, responsible for the compound's anticoagulant activity, which may be significantly reduced by sterilisation but not by deployment (Hårdhammar et al. 1996). Corline® coating uses more advanced technology in which the surface is built upon a macromolecular conjugate of heparin. It is covalently bonded such

that the antithrombin III binding sites are not involved in the covalent bonds but free to interact with antithrombin III (Kristensen et al. 2003, Johnell et al. 2002, van der Giessen et al. 1999, Christensen et al. 2001). In Hepamed® coating, a covalently-bound heparin represents the fourth layer of all covalently bound layers (Vrolix et al. 2000).

Among other antithrombogenic coatings, Seifert et al. (1997) examined properties of surface-immobilised hirudin on polylactide-polyglycolide polymers *in vitro*, and found that the blood-contacting properties of the polymer were improved. Antithrombotic and antirestenotic hirudin/prostacyclin analogue (Iloprost®) coating on metallic stents have also been suggested to exhibit excellent blood compatibility in animal experiments (Alt et al. 2000). Hirudin is a potent blocker of thrombin, the key molecule in the initiation of platelet aggregation and cross-linkage of the fibrin clot. An analog of proctacycline (PGI_2), Iloprost®, accounts for part of the thromboresistance of the intact endothelium and the vessel wall (Alt and Selinger 1998). Other biocompatible stent coatings may be hyaluronic acid, a nonsulfated glycosaminoglycan component of the extracellular matrix (Verheye et al. 2000), and certain anti-platelet agents, such as abciximab (c7E3-Fab, ReoPro™) (Baron et al. 2002).

Antirestenotic stent coatings

Numerous attempts to reduce neointimal overgrowth and restenosis have been performed, yet f ew have clinical relevance. These coating compounds include steroids (De Scheerder et al. 1996b, Lincoff et al. 1997), angiopeptin analogues (de Scheerder et al. 1996a), methotrexate (Cox et al. 1992), forskolin (Lambert et al. 1994, Dev et al. 1995), tyrosine kinase receptor blockers (Yamawaki et al. 1998), fibrin-film coating (McKenna et al. 1998), titanium-nitride-oxide (Windecker et al. 2001), and phosphoryl-cholin (Zheng et al. 1999, Galli et al. 2001, Beaudry et al. 2001, Grenadier et al. 2002), just to mention a few.

Clinically, the most promising antiproliferative stent coatings are paclitaxel and sirolimus (rapamycin). The problem of in-stent restenosis may be greatly resolved by the use of these kinds of coated stents, although there has been some concern of possible hindrance of the vascular healing process at the target area resulting in undesired late complications. Yet, these kinds of negative effects have not been reported up to two year's follow-up after deployment (Degertekin et al. 2002, Regar et al. 2002, Grube et al. 2003, Hong et al. 2003, Sousa et al. 2003, Tanabe et al. 2003, Duda et al. 2002, Schampaert et al. 2004).

4.5. Stent configuration

Stent design appears to affect platelet activation both experimentally and clinically (Kastrati et al. 2000b, Gurbel et al. 2002). Moreover, similarly designed stents with different *rigidity* seem to induce vessel injury and neointimal thickness to a varying extent: more vascular wall injury with increasing rigidity and less with augmenting flexibility (Fontaine et al.1994). Rogers and Edelman (1995) have experimentally tested the effect of design in a rabbit model, and they found that corrugated stainless steel ring stents induced less injury and less neointimal hyperplasia than slotted tube stents. Interestingly, by applying thromboresistant coating to the stents, they were able to reduce thrombosis rates but not intimal hyperplasia, which suggested that flexibility was more important than the coating in the hindrance of neointimal overgrowth. Barth et al. (1996) compared vascular wall reactions between two balloon expandable stents, stainless steel (Palmaz®) and tantalum (Strecker®), with a self-expanding stent (Wallstent®) in external iliac/proximal femoral arteries in a canine model. They concluded that the *lower-hoop-strength* tantalum (Strecker®) stents evoked a greater degree of neointima formation than the higher-hoop-strength self-expanding (Wallstent®) and stainless steel (Palmaz®) stents. The latter two had more pronounced medial atrophy, and in this model the rigid stainless steel (Palmaz®) stent could also penetrate the vessel wall in a flexing artery. Logically enough *mesh size* of the stent influences neointimal growth: when

comparing the same design, the tighter the mesh or the more metal per unit area, the larger is the neointimal growth (Tominaga et al. 1992).

4.6. Problems with stents

One possible complication following stenting is acute or subacute thrombotic occlusion (Sheth et al. 1996b, Gawatz et al. 1997, Orford et al. 2002). In the modern era, however, thrombogenicity of intravascular stents is mainly prevented by efficacious medical therapies. In the future, coated stents may further diminish the rate of thrombosis, and even reduce the need for systemic medication. The treatment of restenosis, related to both progression of the disease and to neointimal proliferation leading to in-stent restenosis, has for a long time been an inevitable problem. It may be greatly facilitated, or even resolved, by the use of novel antiproliferative stent coatings (Morice et al. 2002, Park et al. 2003, Schampaert et al. 2004) and, in the future, by gene therapy (Feldman et al. 2000, Lederman et al. 2002). Although the therapeutic effectiveness of irradiation as a preventive therapy in restenosis has been demonstrated, its role may be receeding along with the success of other anti-restenotic therapies (Tierstein and Kuntz 2001).

Infection, perforation, arteriovenous fistula, and foreign body reactions are previously described complications of stent implantation (Therasse et al. 1994, Hearn et al. 1997, Doslouglu et al. 2001, Yutani et al. 1999, Smith et al. 2001, Korpas et al. 2002). Immunologic reactions are possible but not yet identified with stents. It has also been postulated that the corrosion of metals may cause in-stent restenosis, but its incidence after clinical stenting is unknown (Ryhänen et al.1997, Shih 2001). One suggestion has been that stents may impair late luminal positive remodelling, and therefore, are perhaps not needed beyond six months after implantation (Hoffmann et al. 1996), although some clinical data suggest the contrary (Serruys et al. 1998, Kimura et al. 1996). It is, however, inevitable that by increasing stent implantation, treating longer lesions, and younger

patients, it is possible to create individual inner “full metal jackets”, which may disturb further interventions or operations (Colombo and Karvouni 2000).

4.7. Models in the measurement of PTA and stent-induced vascular response

Reports of vascular response to PTA and stenting in humans are limited by a lack of tissue examination (Farb et al. 2002, Moreno et al. 1996). Animal studies may be used to answer specific biological questions that can help understanding of human pathology (Welt and Rogers 2002). In addition to various rat models porcine coronary, canine coronary/femoral artery, and rabbit iliac artery *in vivo* models have been applied to examine vascular wall reactions to PTA and stenting both in normal and atherosclerotic vessels. Another way of investigating vascular response is via periprocedual venous sampling and/or direct arterial blood sampling from the target area (Mickelson et al. 1996, Neuman et al. 1996).

4.8. Imaging of luminal dimensions in the stented area

Clinically, angiography has still been “the golden standard” whenever post-stenting imaging is required. Intravascular ultrasound (IVUS) has gradually gained popularity in clinical practice, and in experienced hands it has become one of the most reliable methods to visualise luminal and even vessel wall pathology, very often giving more information of the target vessels than conventional angiography (Oemrawsingh et al. 2003). Visualisation of luminal dimensions in a stented area by magnetic resonance angiography (MRA) remains problematic. With current magnetic resonance (MR) scanners, metallic stents are usually safe for imaging, but metals may cause a “black hole” artifact due to magnetic field distortions, and evaluation of luminal patency can be limited (Hug et al. 2000). Therefore, contrast-enhanced computer tomography (CT) can be useful in depicting intraluminal signals of SS stents, whereas tantalum stents may be imaged by MRA (Amano et al. 1999). Artificial narrowing in the nitinol stented area is also possible in contrast-enhanced MRA.

Indirect luminal “visualisation” can also be achieved by different types of flow measurements, often based on ultrasound imaging.

4.9. Experimental models to test stent thrombogenicity

There are several possibilities to investigate stent thrombogenicity *in vitro*. In a steady state model, materials are exposed to various blood components, and in this way basic, material-related differences can be assessed (Zidar et al. 1993). Blood perfusion can be used to mimic *in vivo* situations (Beythien et al. 1994, Beythien et al. 1999). With these kinds of models, the effect of different stent configurations, coatings, and concomitant medication against platelet deposition and coagulation activation can easily be tested. Herrmann et al. (1999) used an elegant *ex vivo* human stasis model to compare differently coated stents, and to assess release kinetics of antithrombotic agents and their effect on platelet adhesion and plasmatic coagulation. There are examples where experimental results have later been confirmed clinically (Hårdhammar et al. 1996, Serruys et al. 1998), while other data have not been validated by later human studies (Wohrle et al. 2001).

5. AIMS OF THE STUDY

The general goal of this study was to evaluate the utility of polylactide (PLA) as a possible stent core material with simple stent prototypes. Additionally, thrombogenicity of uncoated and heparin-coated PLA in comparison with stainless steel was examined.

The specific aims of this study were as follows:

1. to evaluate the rate of biodegradation and long-term tissue responses of poly-L/D-lactide stents in normal rabbit aorta (Studies I-II),

2. to investigate whether PLA disturbs MR imaging after implantation or in the early phase of biodegradation, and specifically, whether it is possible to measure intraluminal dimensions in the stented area by means of Gadolinium-enhanced MR angiography (Study II),

3. to examine whether there are crucial differences in blood compatibility between biodegradable (PLA) and non-biodegradable (stainless steel) stent materials, by challenging the natural anticoagulative factors in static conditions in the presence of foreign material, i.e., a stent strut (Study III),

4. to evaluate how configuration and coating of PLA stents affect their thrombogenicity under *in vitro* blood flow conditions (Study IV).

6. MATERIALS AND METHODS

6.1. Animal studies

6.1.1. Test animals

Forty-four randomly bred, adult NZW rabbits, weighing 2.8-4.9 kg, were used as test animals. In Study I, 20 PLA stent implantations were performed, where as in Study II 12 PLA stents and six stainless steel (SS) stents were implanted. As a control group for magnetic resonance imaging (MRI), six non-operated rabbits were imaged.

The animals received humane care in compliance with the Principle of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals (NIH Publication no. 86-23, revised 1985). The study protocol was approved by the institutional committee for animal research of the Uusimaa Government in Finland.

6.1.2. Anaesthesia

The rabbits were anaesthetised for surgical procedures and imaging studies with a combination of atropine 0.75 mg, ketamine 20 mg, medetomide 300µg, and diazepam 1.0 mg per kilogram body weight, subcutaneously administered. An intramuscular dose of procainepenicillin (150 000IU) served as antibiotic prophylaxis for the surgical procedure. The animals maintained spontaneous breathing during operations. Upon killing, the animals received the same subcutaneous anaesthesia before aortic sampling, and were intravenously administered a high-dose of sodium pentobarbital.

6.1.3. Periprocedual antithrombotic medication

As antithrombotic medication, daily oral acetylsalicylic acid 12.5 mg and ticlopidine 250 mg doses began one to two weeks before the intervention, and continued during the first month after surgery. Subsequently, daily acetylsalicylic acid was administered until the animal was killed. In addition, subcutaneous fractionated heparin (enoxaheparin sodium) was administered three days pre- and post-operatively.

6.1.4. Stents

PLA stents

The PLA 96L/4D stent was designed at the Institute of Biomaterials, Tampere University of Technology, Tampere, Finland. A self-reinforced copolymer of L- and D-lactide (L/D ratio 96/4%) was processed into a double spiral helical stent (length 10 mm, strut diameter 0.2 mm) by Bionx Implants Ltd, Tampere, Finland. The outer diameter of the stent varied from 2.2 to 2.4 mm with an initial lumen of 1.8 to 2.0 mm. Stents were sterilised by gamma irradiation, which results in both reduced molecular weight and decreased degradation rate of the material. After processing and sterilisation, molecular weights of the specimens were 30 372 daltons. Theoretically, the PLA 96L/4D stent immediately begins to expand at body temperature and reaches its maximal expansion of 1.2 fold its original diameter in 24 hours. The final outer diameter was from 2.6 to 2.9 mm.

Tensile strength of the stent strut specimens was tested with a Lloyd LR 30K (Lloyd Instruments, Fareham, England) materials-testing machine. *In vitro* degradation rate of the stent was tested in phosphate-buffered saline. The tensile strength of the strut decreased from 400 Mpa to 135 Mpa in six weeks. After 12 weeks the strength was 19% of the initial strength. The stent strut did not swell during immersion in the buffer solution.

Stainless steel stents

Standard balloon expandable coronary stents (size 2.0-3.5 mm, length 9 mm; NIR™, Medinol, Natick, MA., USA) of stainless steel were applied in Study II for comparison with PLA stents. The thickness of the metallic wire was 0.1 mm.

6.1.5. Surgical procedures

Operation for stent implantation

As the instrument for percutaneous deployment of spiral PLA stents was under development, stents were implanted surgically in all cases. The animals were anaesthetised as described in section 6.1.2. The aorta was exposed retroperitoneally through a left-side longitudinal incision. After systemic heparinisation with an intravenous dose of 150 IU/kg of unfragmentated heparin, the aorta was cross-clamped, followed by a transverse aortotomy.Then, either a PLA stent (Studies I-II) or a SS stent (Study II) was implanted 5 mm proximal to the aortotomy into the infrarenal aorta. The aortotomy was closed with a continuous 8-0 non-resorbable suture (Prolene®). After declamping, an angiography was perfomed in Study I. The incision was closed with continuous absorbable suturing.

Operation for sampling

Upon killing for macroscopic, histologic, and SEM studies, the rabbits were anaesthetised as previously described. A relumbotomy incision was performed, and the abdominal aorta was exposed. The location of the stent was determined visually and by palpation with regard to renal arteries and iliac bifurcation. The animal was then administered a high-dose of sodium pentobarbital, and the abdominal aorta with the PLA stent removed for histologic assessments and SEM.

6.1.6. Imaging studies

Angiography

Patency of the vessel after PLA stent implantation was determined in Study I by a lateral intraoperative angiography (contrast media: Omnipaque® 180 mg/ ml, one projection). Before termination, an angiography was performed in the same way.

Magnetic resonance imaging

All rabbits in Study II were scanned with a Siemens Vision 1.5 T MRI Scanner (Siemens, Erlangen, Germany) with the aid of a quadrature knee coil. The abdomen was first imaged in the axial and coronal directions with a T1-weighted sequence (TR 760, Te 12, slice thickness 3 mm, 160 x 256 matrix size) to localise the abdominal aorta and the stented area. The infrarenal aorta was then imaged with a 24-sec 3 d flash sequence (Slab/Eff. thickness 47/1.8 mm, one acquisition, matrix size: 180 x 256), first without a contrast medium and then three times immediately after an intravenous dose of 0.3 mmol/kg gadolinium (Dotarem®, Guerbet, Villepinte, France) to achieve the best possible contrast media concentration in the infrarenal aorta. Proximal (1 cm below the left renal artery), distal (immediately above the bifurcation), and narrowest infrarenal aortic luminal diameters were established with the MR scanner by computerised measurements. The longitudinal length of the stent artifact was measured in the same manner.

6.1.7. Histologic assessment

The stented aorta was cut horizontally, and one half of the stented area was fixed with 4% formalin solution for histologic examination, the other half sampled for SEM. Specimens of formalin-fixed aortas were buffered overnight in 10% phosphate-buffered neutral formalin. The aortas were then imbedded in paraffin through a process of graded concentrations of ethanol and xylene, and sectioned with a microtome producing 3 µm-thick sections. After rehydration with xylene and

graded concentrations of ethanol, the samples were finally stained with hematoxylin and eosin, van Gieson, or elastin stainings.

Staining for elastin enables the assessment of elastin fibers in the tunica media, while van Gieson staining determines the relative amounts of fibrous and smooth muscle proliferation of the neointima. The van Gieson dye reveals smooth muscle proliferation of the neointima by staining smooth muscle orange and collagenous fibrous tissue red.

6.1.8. Scanning electron microscopy

The other half of the stented area of the aorta was fixed in 2.5% buffered glutaraldehyde solution for SEM. After fixation, the tissue samples were dehydrated in ethanol and critical-point dried (BAL-TEC Ltd., Balzers, Liechtenstein), mounted on aluminium studs, and coated with gold by means of a Jeol JFC-1100 sputtering device (Jeol Ltd, Tokyo, Japan). The samples were then examined with a Jeol JSEM-820 scanning electron microscope (Jeol Ltd, Tokyo, Japan) at 4-20 kV acceleration voltage.

6.1.9. Statistical analysis

The numerical data are expressed as mean ± SD (Study II). Variations in the measured indices between three groups (PLA- or stainless steel-stented animals, and non-stented animals) of the various assessment points (proximal, distal, and narrowest aortic dimensions) were analysed by the non-parametric Kruskal-Wallis one-way analysis by ranks (Statistica^{TM} v. 5.5, StatSoft Inc., Tulsa, OK, USA). Non-parametric analysis was chosen due to small sample sizes. The rank sums were then used for Dunn's test at a significance level of 5% (Medstat, Astra Group A/S, Copenhagen, Denmark). Values of $p \leq 0.05$ were considered statistically significant**.**

6.2. *In vitro* studies

The goal of the *in vitro* studies was firstly to challenge the native anticoagulative factors of human blood in the presence of a stent strut, with a steady state model under static conditions. Unheparinised and heparinised PLA struts were compared with stainless steel (Study III). To assess the procoagulant capacity of platelets, a range of platelet numbers with and without plasma was provided. In the measurement of coagulation activation, various crucial markers of the coagulation cascade were determined: prothrombin fragments 1+2 (F1+2), thrombin-antithrombin compex (TAT), fibrinogen, and thrombin time (TT) (Figure 3). Next, a whole blood perfusion model was applied to evaluate the effect of stent design and coating on blood compatibility of PLA stent prototypes (Study IV). Furthermore, to mimic the pathophysiology of balloon injury by the collagen exposure, the stent-containing segment of the perfusion tubing was precoated with type I collagen in the last series of these experiments.

6.2.1. Stent materials and stents

The Institute of Biomaterials, University of Technology, Tampere, Finland produced all biodegradable stent struts and stents, and the heparinisation was provided in cooperation with Helsinki University of Technology, Helsinki, Finland. The heparinsodium, derived from porcine intestinal mucosa, was provided and analysed by the Sigma Corporation (St. Louis, MO, USA). Before the experiments, all biodegradable stent struts and stents were sterilised by gamma irradiation.

Stent materials

One cm stent struts of each material were examined. Stent struts of stainless steel were cut from commercially available stainless steel coronary stents. The diameter of the wire was 0.1 mm. The PLA stent strut was made of a self-reinforced copolymer of L- and D-lactide (L/D ratio

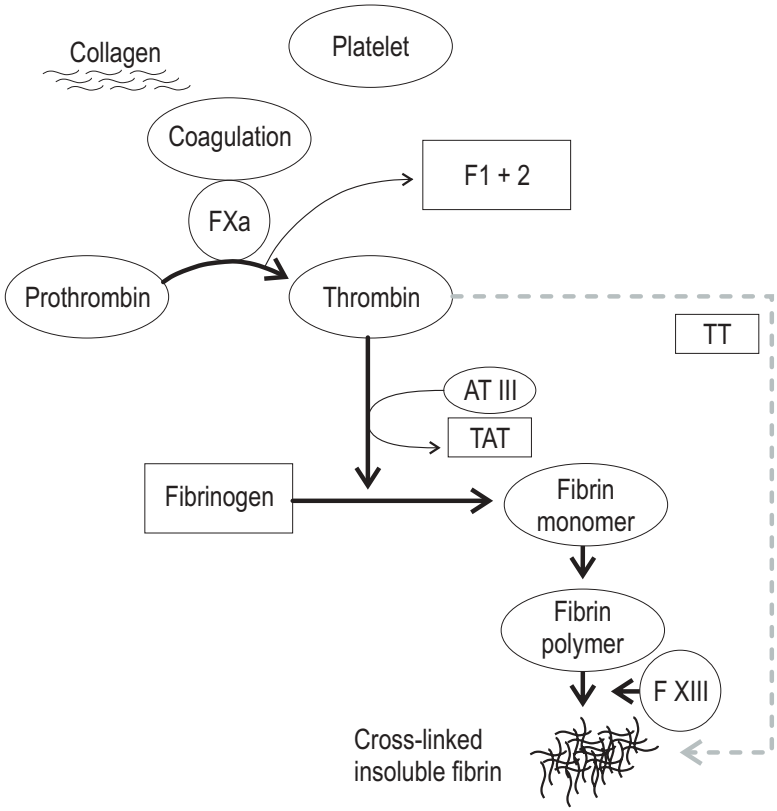

Figure 3. Schematic, simplified presentation of platelet response and coagulation activation relevant to the present study. The markers measured are in square boxes. The cascade of fibrinolytic mechanisms is not shown.
ATIII = Antithrombin III, F1+2 = Prothrombin fragments 1+2, FXa = factor Xa, FXIII = Factor XIII, TAT = Thrombin-antithrombin complex, TT = Thrombin time

96/4%). By a special die-extrusion coating process, at a temperature of 60°C, the melt coating material, a heparin-blended (5801 IU/g) copolymer of ε-caprolactone and L-lactide (Hepa P(CL95/L-LA5, molecular ratio of ε-caprolactone to L-lactide: 95:5), was extruded directly onto the

PLA strut to obtain Hepa P(CL95/L-LA5)-PLA stent struts. The diameters of the PLA and Hepa P(CL95/L-LA5)-PLA struts were 0.2 mm and 0.3 mm.

Stents

In Study IV, seven different biodegradable stents of two types of configurations, either self-expanding (double spiral) or partly self-expanding/partly balloon-expandable (braided), were examined. Standard balloon-expandable coronary stents (size 3.0-3.5 mm, length 10 mm, wire diameter 0.1 mm, NIR^{TM}, Medinol Ltd, Natick, MA, USA) of stainless steel were used as controls for each series of perfusion.

Double spiral PLA stents

A self-reinforced copolymer of L- and D-lactide (L/D ratio 96/4; PLA96L/4D) was processed into a self-expanding double spiral helical stent. The maximal outer diameter of the stent before vs. after expansion was 2.5 vs. 3.0 mm when uncoated and 3.0 vs. 3.6 mm when coated. The length of each stent was 10 mm.

Braided PLA stents

Self-reinforced PLA 96L/4D stent struts were braided into the form of a partly self-expandable, partly balloon-expandable stent consisting of 16 struts. The braids were heat set on metal mandrels having a diameter of 3.2 mm and cut to a length of 10 mm.

The spiral PLA stents were heparinised by the die-extrusion coating process, a heparin-blended copolymer of ε-caprolactone and L-lactide [Hepa P(CL/L-LA)] serving as coating material. The heparin coating of braided PLA stents were performed either by die-extrusion, or by using acetone as dissolvent and a copolymer of ε-caprolactone and D,L-lactide [P(CL/D,L-LA)] as coating

polymer. The original heparin potency before all processing was 179 ± 4 USP units/mg. The coating of stents with P(CL/D,L-LA) was performed in the same manner without heparin.

6.2.2. Assessment of stent-attached heparin

The amount of heparin leaching from the stent strut and stents was assessed with the Blyscan glycosaminoglycan assay® (Biocolor Ltd., Belfast, Northern Ireland). The strut-attached heparin was measured before and immediately after the experiment (Study III). Three parallel heparinised stent struts were incubated as described in section 6.2.4. for platelet deposition or coagulation activation experiments. The heparin content before the incubation was 3.80 ± 0.30 µg/strut (mean 40.00 µg/cm^2). At the end of the platelet deposition assay, it was reduced to 0.15±0.03 µg/ strut (mean 1.58 µg/cm^2), and after coagulation activation assessment to 0.13 ± 0.05 µg /strut (mean 1.42 µg/cm^2).

Assessed by the same assay (described above), the estimated heparin content per stent was 8.7 µg for the spiral coated PLA stents, 6.0 µg and 8.3 µg for the two Hepa P(CL/L-LA)-coated PLA stents, and 11.4µg for the braided P(CL/D,L-LA)-heparin-coated PLA stents before perfusion (Study IV).

6.2.3. Blood collection and preparation

The Institutional Review Board of the Wihuri Research Institute approved the study. Blood was donated by 40 healthy volunteers who had not taken any medication during the previous 10 days and who had been fasting 8 h before sampling. After resting for 15 minutes, the blood samples were obtained with a free-flowing technique with the first 3 ml discarded. Samples were collected through venipuncture from the antecubital vein via an inserted polytetrafluoroethylene cannula

(Viggo®) into polypropylene tubes. Platelet counts and haematocrit were determined with a Thrombocounter Coulter T-540.

In Study III, for gel-filtered platelets (GFP), six volumes of free-flowing blood were collected into one volume of acidic citrated dextrose, pH 4.5. Nine volumes of blood were collected into one volume of either 1) 3.2% sodium citrate for plasma specimens or 2) 30 µmol/l D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone (PPACK) for platelet-rich plasma (PRP) specimens in platelet deposition studies, or 3) 0.5 µmol/l or 0.75 µmol/l PPACK for PRP and platelet-poor plasma (PPP) for the assessment of coagulation activation. PRP was separated from blood by centrifugation (180 x g, 12 min, 22ºC). PPACK (Calbiochem-Novabiochem Corp, San Diego, CA, USA) is a direct thrombin inhibitor without chelation of ionic calcium or magnesium necessary for platelet adhesion receptors.

In Study IV, nine volumes of blood were collected into one volume of 30 µmol/l PPACK for preparing PRP, which was separated from blood after centrifugation in the same manner as in Study III. Platelets in PRP were labelled with 0.45 µl of 5-hydroxy[G-^{3}H]tryptamine creatinine suphate /ml PRP (^{3}H-serotonin, 10 nmol/l, Amersham Pharmacia Biotech UK Limited, Buckinghamshire, England, UK) by 15 min incubation at 37ºC. Before the perfusion, the labelled PRP and the remaining blood cells were recombined and incubated at 22ºC for 30 min as previously described (Mustonen and Lassila 1996). $CaCl_2$ was added to the priming solution (phosphate buffered saline, PBS) at 0.8 mmol/L and $MgCl_2$ at 0.5 mmol /l. For scanning electronmicroscope examination, all the same procedures were performed with the exception of platelet labelling.

6.2.4. Platelet deposition analyses (Study III)

GFP was prepared from PRP after a single washing step in the presence of prostaglandin E1 (25 ng/ml) and apyrase (1 U/ml) (Sigma), and the platelet suspension was then passed through a Sepharose CL-2B column (Pharmacia LKB) and eluted to Hepes buffer without divalent cations. Platelets were labelled with ^{3}H-serotonin at a concentration of 10 nmol/l at 37°C for 15 min. The labelled platelets were adjusted to 100, 300, or 500 x 10^6/ml of Hepes buffer, supplemented with Ca^{2+} (2 mmol/l) and Mg^{2+} (1 mmol/l) just prior to the assay. The stent struts were placed in flat-bottom multidishes (NUNC®) precoated with 2% human serum albumin (HSA) and loaded with 0.5 ml of the various platelet suspensions. The suspensions were: two densities of GFP, 300 x 10^6/ml and 500 x 10^6/ml, representing normal and supranormal platelet densities; GFP 500 x 10^6/ml diluted 1:1 with 3.2% platelet-poor plasma (PPP); and PRP anticoagulated with PPACK either at 100 x 10^6/ml or at 300 x 10^6 platelets/ml. After a 20 min incubation at 37°C with a slowly rotating motion (70 rotations per minute), the struts were removed, washed three times by dipping into PBS, and the ^{3}H-serotonin activity of the stent struts measured in a liquid scintillation counter (Rackbeta, Wallac, Turku, Finland). The number of platelets deposited on the strut area was calculated from the number of platelets added and from their specific activity. Additional samples of GFP-incubated struts (at both GFP densities, 300 x 10^6/ml and 500 x 10^6/ml) were fixed with 2.5% glutaraldehyde solution for SEM.

6.2.5. Coagulation activation (Study III)

PRP was prepared as described and finally adjusted to a platelet density of 300 x 10^6/ml. Limited anticoagulation was provided by PPACK at two final concentrations: 0.5 µmol/l and 0.75 µmol/l. The concentrations of Ca^{2+} and Mg^{2+} were physiological. The stent struts were placed in HSA-coated flat bottom multidishes, and loaded with 1 ml of either of the four different PRP/PPP suspensions. As controls, the same measurements were performed for incubated PRPs and PPPs

without a stent strut. After 20 min incubation (37°C, 70 rotations per minute) the reaction was halted by putting the samples on ice. Thrombin time (TT) was measured in the plasma (PRP or PPP) in which the stent struts had been incubated with a Coagulometer Amelung KC 1 A (Heinrich Amelung GmbH, D-4920 Lemgo 1-Lieme, Germany). Likewise, fibrinogen was assessed by the functional method of Clauss, and enzyme-linked immunosorbent assays (ELISAs) were applied to determine prothrombin fragments 1+2 (F1+2) and thrombin-antithrombin complex (TAT) (Dade Behring, Marburg, Germany). The upper limit for the follow-up of TT values was 300 seconds.

6.2.6. Description of the whole blood perfusion model (Study IV)

Stents were implanted with appropriately sized balloon catheters into a polyvinylchloride (PVC) tube (Ø3.2 mm, Sorin Biomedica, Espoo, Finland), with or without precoating the tube with fibrillar type I collagen (Collagen Reagent Horm, Nycomed Pharma GmbH, Unterschleissheim, Germany). Each experiment was repeated five times using blood from different individual donors. The order of a stent perfused inside each series was randomly chosen. Blood from 25 individual donors was used for the following perfusion comparisons, and from four donors for representative SEM:

Pilot group:

A stainless steel stent was compared with a PVC-tube without a stent and with a spiral PLA stent.

Study groups (I-IV):

A stainless steel stent was compared with

I) a spiral PLA stent and a spiral PLA stent coated with a heparin-blended copolymer of ε-caprolactone and L-lactide [Hepa P(CL/L-LA)]; (heparin dose: 8.7 µg/stent),

II) a braided stent with Hepa P(CL/L-LA)-coating (heparin dose: 6.0 µg/stent) and a braided stent with Hepa P(CL/L-LA)-coating (heparin dose 8.3 µg/stent),

III) an uncoated braided PLA-stent, a copolymeric ε-caprolactone and D,L-lactide [P(CL/D,L-LA)]-coated stent, as well as with a P(CL/D,L-LA)-heparin-coated stent (heparin dose:11.4 µg / stent), IV) stents with the same design and coating as in III), but the stent-containing segment of the PVC-tubes was precoated with fibrillar type I collagen before implantation of the stents.

The total length of the closed perfusion tubing was 83 cm. The temperature of the primer, the blood, and the stent-containing segment were stabilised in a water bath at 37ºC for 5 min. The perfusion was initiated with cation-containing PBS (30 s), and continued with a roller pump at a flow rate of 30 ml/min for 90 s. To finish the perfusion, PBS-rinsing was repeated (30 s) and the stent-containing segment of the tube was cut and rinsed with PBS. The deposited platelets with their ^{3}H-serotonin activity were measured as described in section 6.2.4. Before and after perfusion, plasma ^{3}H-scintillation (serotonin release), and total blood ^{3}H-scintillation activities were determined as described elsewere (Mustonen and Lassila 1996). To assess the extent of coagulation activation, prothrombin fragments F1+2 were measured just prior to the perfusion and again after 75 seconds of blood perfusion by ELISA, as in section 6.2.5.

6.2.7. Scanning electron microscopy (SEM)

The morphology of the adhering platelets on the different stent struts and stents were investigated qualitatively by SEM. In Study III, after incubation with GFP at a platelet density of either 300 x 10^{6}/ml or 500 x 10^{6}/ml, the stent struts of the different materials were rinsed with PBS. After fixation with buffered glutaraldehyde solution (2.5%), the struts were dehydrated in ethanol and critical-point dried (BAL-TEC Ltd, Baltzers, Liechtenstein), mounted on aluminum studs, and coated with gold. To qualitatively investigate the morphology of adhering platelets on the different stents in Study IV, the perfused stents were washed as described above with PBS and fixed with buffered glutaraldehyde solution for 90 min and then immediately with 1% osmiumtetroxide for 60

min. Thereafter, the stents were dehydrated in ethanol and critical-point dried, mounted on aluminum studs, and coated with platinum. Scanning electron microscopic analyses were performed with a Jeol JSEM-820 Scanning Electron Microscope (Jeol Ltd., Tokyo, Japan; in Study III) and a Zeiss Digital Scanning Microscope 962 (Oberkochen, Germany; in Study IV).

6.2.8. Statistical analyses

All numerical data are expressed as mean $\pm$ SD The variation in the measured indexes between the different stents struts and stents were analysed by non-parametric analysis (Kruskal-Wallis one-way analysis), and the Dunn's test, as in the animal studies (described in section 6.1.9).

7. RESULTS

7.1. Tissue compatibility and biodegradation of intravascular PLA stents

In general, PLA stents were well-tolerated in long-term follow-up in the rabbit aorta model. 25/26 aortas remained patent through the planned follow-up periods (Studies I and II). The minimal tissue response consisted of mild inflammatory reaction upto six months after implantation, and a formation of fibrous neointima, but in one aorta sampled at one year, a persistent inflammatory reaction was found. No fragmentation of the material occurred at any time point, and each stent was gradually replaced by fibrosis in two years.

Histologic assessment

At one month after stent implantation (n = 2) regular endothelial lining was detectable in the stented aorta. Neointima surrounded both sides of the stent spiral. A mild inflammatory reaction in the vessel wall consisted of lymphocytes and occasional macrophages, with no signs of neutrophilic granulocytes or eosinophils. In the aortas sampled at two to three months after implantation (n = 5) the histologic situation was essentially unchanged, with the exception of one aorta with chondroid metaplasia with a mural thrombus and a luminal reduction of approximately 30 % in the same area.

By six months after stent implantation (n = 3) the inflammatory reaction in the vessel wall had decreased. Fibrotic tissue surrounded the stent material, and the muscular layer of the media appeared to have thinned in the stented area, with the stent itself showing no fragmentation. Local hemosiderin accumulations, and few vessels with red blood cells (neovascularisation) were detectable in the fibrotic area. In Study II, one rabbit suffered from aortic thrombosis at this time point, and had to be euthanized. According to autopsy results, thrombosis was probably due to partial opening of the double spiral helical structure, presumably at the time of implantation.

9-12 months after the implantation (n = 11) hydrolysation of the stent was histologically evident, with no signs of fragmentation. A mild foreign body reaction with scarce giant cells and some macrophages was evident in 4/11 sampled aortas, but one aorta had a persistent inflammatory reaction with lymphocytes, macrophages, and very few giant cells. Some calcium deposits were detectable in the fibrotic tissue, and some vessels were evident both in the neointima and the muscular layers. In one aorta, the area of calcium deposits resembled the chondroid metaplasia, previously found in another aorta sampled at three months after implantation.

Degradation of the stent had proceeded with some cells visible inside the material from 20 to 22 months after implantation (n = 2). Endothelium was intact, and fibrosis resembled that as at one year with haemosiderin-containing macrophages and calcium accumulation in the neointima. Sparse areas of fibrosis showed smooth muscle cell proliferation. From two years after implantation (n = 3), the material appeared largely hydrolysed and was no longer detectable under polarising light, as at earlier time points. Fibrotic proliferation was less active than earlier, and no signs of inflammation were evident. Mild smooth muscle cell proliferation was detected in the fibrous tissue. Some areas of the muscular layer were thinned resulting in good luminal preservation. Calcifications and haemosiderin accumulations were evident as at previous assessment points.

Scanning electron microscopic assessment

Similar to the histologic findings, endothelium appeared to cover the stented area by one month after implantation, which was also true at later time points. The stent seemed to be well-fitted subendothelially, with the lumen retaining its patency.

7.2. Assessment of luminal diameters after PLA-stenting by MRA

Stainless steel caused a signal loss in the stented area, as expected, making evaluation of luminal

diameters impossible. In contrast, PLA stents caused no magnetic field distortion, allowing detection of luminal patency in all 12 animals. In the follow-up group of PLA-stented rabbits, 5/6 survived through to the end of the 12 month follow-up. PLA-stenting allowed assessment of luminal dimensions at various time points up to one year. The exact location of the PLA stents, in regard to renal and iliac arteries, could only be established at necropsy. The proximal dimensions (1 cm below the left renal artery) of the control and those groups studied immediately post-operatively did not differ, while the distal dimensions (at aortoiliac bifurcation) were narrower in both groups implanted with a stent. This difference was significant only between non-operated controls and those implanted with a PLA stent ($p < 0.01$). The narrowest region of the aorta was generally in the stented area, and probably at the line of suturing. No significant differences in aortic measurements emerged during the follow-up period, with a non-significant tendency for normalisation of the narrowest diameter towards the end of the study.

7.3. Material-related haemocompatibility of PLA

In Study III, a steady state model was used to assess possible material-related rheological differences between stainless steel, uncoated PLA, and heparin-coated PLA. In general, in comparison with stainless steel, both uncoated PLA and heparin-coated PLA stent struts appeared to possess favourable blood compatibility.

Platelet deposition on stent materials in different platelet suspensions

The stainless steel strut accumulated significantly more platelets than did the PLA strut, with a physiological platelet count of 300×10^6/ml ($p < 0.05$). Although a range of platelet numbers, with and without plasma, were provided to assess the preocoagulant capacity of platelets, the numbers of adhering platelets on SS, PLA, and hepa-P(CL95/L-LA5)-PLA stent struts did not differ statistically.

At representative SEM assessment, platelets appeared mostly to be uniformly spread or deposited in small aggregates with detectable pseudopodia. On the surface of stainless steel stent struts, platelets often presented full spreading and likely activation (“fried-egg” appearance), in contrast to platelets on PLA surfaces, which only changed shape. Although platelets on heparin-coated PLA appeared to show the least extent of pseudopodia formation in general, in occasional samples more platelets were visible on these stent struts than on either SS or PLA.

Coagulation activation in PRP/PPP on stent materials

In no assessment did fibrinogen content in plasma incubated with or without a stent strut differ. Due to limited anticoagulation with the low concentration of PPACK, thrombin -antithrombin complex (TAT) revealed varying levels of coagulation activation, with control values (i.e. values for incubation suspensions without a stent strut) similar to PLA. In the assessments in which platelets were in contact with stainless steel, TAT values tended to be higher. However, none of these differences were statistically significant. Similarly, with prothrombin fragments 1+2 (F1+2), a tendency for the highest values to occur with stainless steel and the lowest with Hepa P(CL95/L-LA5)-PLA was observed in all assessments. This difference was significant only at a low concentration of PPACK in PRP, under the conditions prone to generate the most thrombin. Control values most closely resembled PLA in their generation of F 1+2.

Under all conditions, thrombin time (TT) was significantly prolonged by the hepa-P(CL95/L-LA5)-PLA in comparison with TT for stainless steel. The difference in TT between the PLA and Hepa P(CL95/L-LA5)-PLA reached statistical significance in the presence of platelets employed in PRP at 0.5 µmol/l PPACK, or in PPP at 0.75 µmol/l PPACK.

7.4. Blood compatibility of PLA stents in a whole blood perfusion model

In any assessment, the least platelet deposition occurred on stainless steel stents ($p < 0.02$). In addition, coating with a heparin content of 8.3 μg or more/stent appeared to diminish the number of adhering platelets, but due to interindividual variation, these differences were not significant. Among all biodegradable stents, the double spiral PLA stent attached more platelets than did the braided P(CL/D,L-LA)-PLA stent coated with heparin ($p < 0.02$). The most platelets were deposited on double spiral PLA stents compared with either PVC-tube without a stent ($p < 0.01$) or SS stents ($p < 0.02$). When comparing SS stents with two braided PLA stents with different heparin-coatings, the braided PLA stent with a heparin-content of 6.0 μg/stent collected more platelets than SS ($p < 0.01$). The uncoated braided PLA stents collected the most platelets, when uncoated, P(CL/D,L-LA)-coated , and P(CL/D,L-LA)-heparin-coated braided PLA stents were compared with SS stents ($p < 0.01$). The plain P(CL/D,L-LA)-coating without heparin on braided PLA stents appeared to reduce the amount of platelets almost as effectively as P(CL/D,L-LA)-heparin, although not significantly.

To mimic the pathophysiology of balloon injury related to PTA, the stent-containing segment of the PVC tubing was coated with fibrillar type I collagen. In general, the number of depositing platelets on any stent was augmented 10 to 30 fold compared to perfusions without collagen. Due to large interindividual variation, PLA and P(CL/D,L-LA)-heparin-coated PLA did not differ in their capacity to attach platelets, although among biodegradable stents there was a tendency towards the heparin coating reducing platelet deposition. The only significant value was between plain P(CL/D,L-LA)-coated stents and SS, the latter again collecting the fewest platelets ($p < 0.01$). Irrespective of the presence of heparin or collagen, coagulation activation, measured by F1+2, was not systemically activated before or after the perfusion. Representative SEM revealed no signs of fibrin accumulation on any of the stent materials in the absence of collagen, but in the presence of

collagen platelet-associated local fibrin accumulation was detected on all stent materials. Heparin-coating appeared to diminish this fibrin deposition.

Morphologic findings in SEM

In accordance with the quantitative data, more platelets deposited on biodegradable stents than on stainless steel stents in general (Figure 4). As indicated by reduced pseudobody formation and platelet spreading, there appeared to be less platelet activation on braided PCL-PLA-heparin-coated PLA stents than on uncoated braided PLA stents. In occasional samples the metallic surface showed more ballooning membranes than did any other stent. Platelet aggregates of varying size were detectable on all stent surfaces, with barely enmeshed fibrin. As in the quantitative analysis, the number of platelets deposited in the presence of collagen was clearly higher than in its absence.

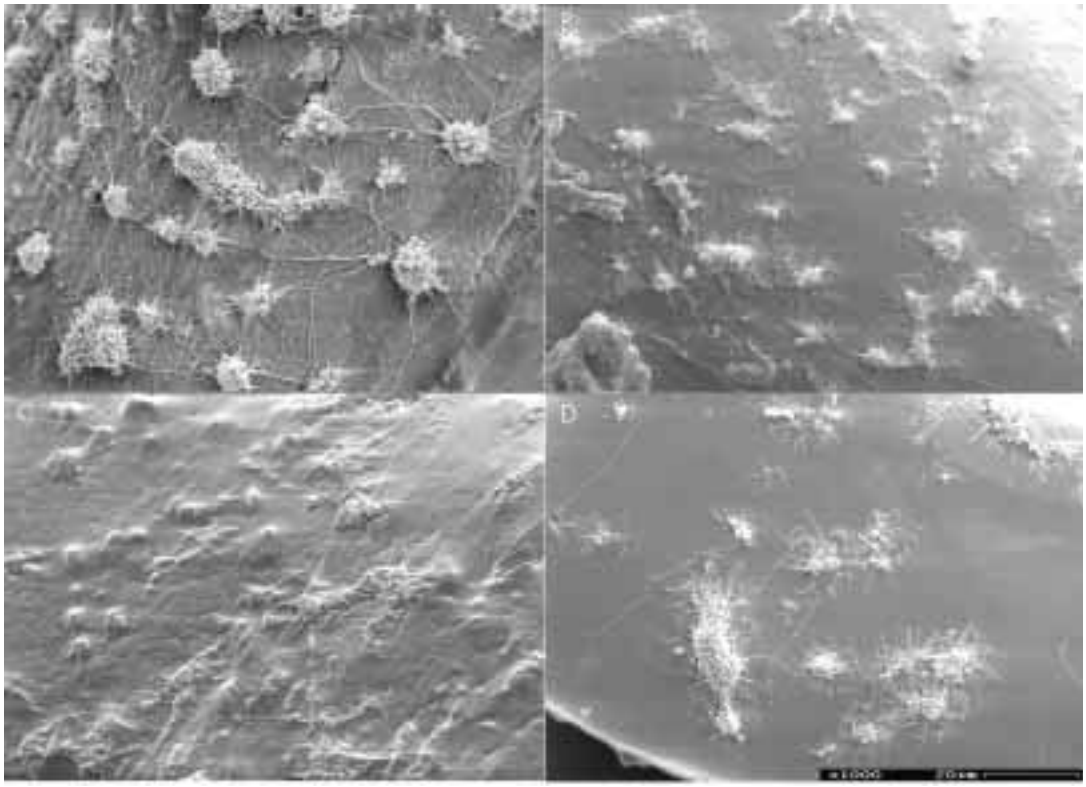

Figure 4. Representative SEM after whole blood perfusion. The stent-containing segment of the perfusion tube was precoated with fibrillar type I collagen. Platelet deposition presented on A) uncoated braided PLA stent, B) polycaprolactone-D,L-lactide coated PLA stent, C) polycaprolactone-D,L-lactide-heparin coated PLA stent, and D) stainless steel stent. Magnification 1000x. Bar in D= 20µm.

8. DISCUSSION

8.1. Materials and methods

In order to assess long-term vascular response after stenting, an adult rabbit model was chosen, because the animal studies were planned to last at least two years, and therefore a porcine model would have been more difficult to manage. A miniswine model would have been an alternative. As the aim was to assess the effect of intravascular biodegradation of the material on vascular wall, normal rather than atherosoclerotic vessels were examined. With the lack of a percutaneous deployment system for the double spiral stent, the PLA stents were implanted through a surgical retroperitoneal approach, thus the vascular trauma induced differed from PTA injury. However, the stents were implanted proximal to arteriotomy, into an area of non-denuded endothelium.

The possible effect of early biodegradation of PLA stents on magnetic resonance imaging was assessed in Study II. Non-operated animals and stainless steel (SS) stented animals were compared with PLA-stented rabbits. Other types of stents (e.g. tantalum or nitinol) could also have been chosen, as the artifact size may have been smaller than with SS stents. In addition, all animals could have been imaged prior to operation to obtain individual reference measurements. Such a protocol was not employed, as the aim of the study was to examine the effect of biodegradation, not surgery, on MR imaging. Furthermore, another reason was to limit the number of experiments per test animal. For the same reason, conventional follow-up angiographies were not used for comparison with MRA. In urologic applications, barium sulphate has been used to mark the PLA stent edges, but as this agent is not suitable for vascular application and no other marking systems were available for PLA stents, aortic measurements were performed by assessing proximal (1 cm below the left renal artery), distal (at aortoiliac bifurcation), and narrowest luminal diameters at the stented area.

Material related differences in haemocompatibility were examined in a steady-state model (Study III). Signs of platelet deposition and coagulation activation were measured, and the effect of plasma components on platelets and coagulation was assessed. Others have used the same kind of models with bovine (Zidar et al. 1993) and human blood (Seifert et al. 1997). Beythien et al. (1994) and Gutensohn et al. (1997) used *in vitro* perfusion models without collagen precoating of the perfusion tubing in the assessment of stent thrombogenicity, but other models (*ex vivo* human stasis model) have been applied as well (Herrmann et al. 1999). To mimick the *in vivo* situation a whole blood perfusion model was designed (Study IV). As endogenous collagen exposure after PTA and stenting is known to enhance thrombotic events, collagen precoating of the perfusion tubing was chosen as a natural thrombogenic component of the vessel wall.

8.2. Tissue compatibility and biodegradation

The experimental assessment of biocompatilitity of degradable intravascular materials remains highly challenging. Cell toxicity testing of new biomaterials can be assessed *in vitro*, but the assessment of tissue compatibility is more complex, as reactions may depend on many factors, including the extent of induced vascular injury, periprocedual medication, and deployment techniques. Especially, after the onset of stent degradation, polymer-specific induction of vascular response becomes pivotal (Zidar et al. 1994, van der Giessen et al. 1996, Yamawaki et al. 1998). In addition, the extent of tissue response appears to greatly depend on degradation time of the material (Gogolewski et al. 1993, Lincoff et al. 1997). Tissue reactions evoked by a biodegradable material thus remain a target-specific issue, which must be taken into account when data from another species is interpreted and brought into the discussion of human pathological conditions.

Previously, experimental models have demonstrated that a vascular wall reaction depends strongly on the severity of the injury caused by metallic stent implantation in normal animal arteries

(Schwartz et al. 1992), and intimal thickening can be avoided by preservation of endothelium (Fishman et al. 1975, Clowes et al. 1983, Stemerman et al. 1977).When PTA and/or, experimental endothelial denudation, are used, metallic stents may induce severe chronic inflammation (De Scheerder et al. 1997, Barth et al. 1996), and granulomatous or significant foreign body reaction (Farb et al. 1999, Kornowski et al. 1999). In the current rabbit aorta model, tissue reactions were assessed in 26 animals, with 15/26 rabbits followed up for at least 12 months. All but one remained patent through the long-term follow-up, and in two aortas there was a chondroid metaplasia in the vessel wall; in one of these cases causing mild luminal reduction at three months after implantation. The same kind of rare condition has previously been described in humans related to long-term venous catheterisation (Dvorak et al. 1999). Only a mild inflammatory reaction was present in all aortas sampled up to six months. From that point, inflammatory reaction declined with proceeding biodegradation and neointimal formation, but one aorta presented a persistent inflammatory reaction at one year after stent implantation. All other (16) aortas sampled after the onset of biodegradation ($\geq$ 9 months) presented mild or no signs of inflammatory reaction in the vessel wall with advancing stent disintegration.

Poly-L-lactide (PLLA) has been well tolerated in a dog femoral artery model, and a mild tissue reaction similar to that in the current series has been described (Zidar 1994). Instead, polyglycolic acid (PGA) stents, which degrade much faster (in 6 weeks), evoke strong vessel wall reaction with possible serious complications, and therefore pure PGA stents do not appear recommendable for vascular applications as stent core material (Susawa et al. 1993). As it has earlier been pointed out, increasing degradation time may positively correlate with tissue compatibility, presumably because gradual, slow degradation reduces both the immune and the inflammatory responses. Similarly, stents coated with higher molecular weight PLLA were associated with less severe vascular response and acute and chronic inflammation than stents coated with lower molecular weight PLLA

in a porcine model (Lincoff et al. 1997). The present investigation suggests that poly-L/D-lactide stents with a final molecular weight (after sterilisation) over 30 000 Da are quite well tolerated in rabbit arteries. However, whether this holds true in human vascular tissue awaits further research. In the future, tailoring stent manufacture, affecting degradation time by searching for new processing and sterilisation methods, and producing copolymeric core materials may help in minimising the vascular wall reactions due to biodegradation (Blindt et al. 1999).

Nowadays, it is well accepted that the development of restenosis greatly depends on the result of complex interactions with activated platelets, injured endothelial cells, leukocytes, inflammatory chemokines and cytokines, and endothelial growth factors, with inflammation playing a pivotal role linking early vascular injury to neointimal hyperplasia and late lumen loss (Weyrith et al. 2002, Sousa et al. 2002, Welt and Rogers 2002). After coronary stent placement in humans, neutrophils are found surrounding the struts in the early phase, but also macrophages and lymphocytes are detectable. After the first month, neutrophils are absent but chronic inflammatory cells (macrophages and lymphocytes) are present, even at six months after the procedure (Farb et al. 1999). Furthermore, the severity of inflammation is associated with the underlying arterial wall morphological status: stent struts penetrating necrotic lipid-rich plaques, or related with damaged media cause more severe inflammatory response than struts in contact with fibrous plaques. The long-term ($\geq$ 9 months after stent implantation) postmortem data has demonstrated that disruption of the arterial media and lipid core penetrating stent struts correlates with chronic inflammation, and finally with greater neointimal growth (Farb et al. 2002). Interestingly, peristrut neoangiogenesis, first described in humans in the same report, appeared to be strongly associated with inflammation, but not with neointimal thickness. The authors suggested, on the basis of former studies (Wysocki et al. 1998, Hausner et al. 1999, Gill et al. 2001, Couper et al. 1997, Brasen et al. 2001), that neovascularisation may have been stimulated by vascular endothelial growth factor (VEGF)

released by inflammatory cells, and the presence of neovessels could offer a potential target for antirestenotic therapies. In Studies I and II, scarce vessels in the neointima as a sign of neovascularisation were detectable in samples from six months after stent implantation, but they did not seem to be particularly associated with inflammation. As normal, non-atherosclerotic aortas were examined after implantation of a slightly self-expandable stent in an area of intact endothelium, no further conclusions can be drawn from the current results.

8.3. Magnetic resonance imaging after PLA stenting

Contrast enhanced magnetic resonance angiography has become a highly accurate method of diagnosing vascular abnormalities of the thoracoabdominal and peripheral vessels (Joarder and Gedroyc 2001). Its use in the assessment of supra-aortic and coronary artery lesions is on the increase (Loewe et al. 2004, Maintz et al. 2004). Due to magnetic field distortions, stents cause considerable artifact and therefore evaluation of luminal patency often remains limited in the stented area (Amano et al. 1999, Hug et al. 2000, Lenhart et al. 2000). One assumption in the beginning of the current study was that the implanted PLA stents would be detectable by high field (1.5 T) MR scanners, as are orthopaedic PLA screws with a molecular weight of 50 000 Da (Lohman et al. 1999). Although this was not true for PLA stents, evaluation of luminal diameters throughout the study, up to the early phase of biodegration, was clearly possible. Furthermore, the measurements in the PLA-stented follow-up group appeared to be reproducible: no differences in aortic dimensions emerged during 12 months' follow-up, although there was a non-significant tendency towards luminal normalisation in the narrowest area with advancing stent hydrolysation. The pixel size of the scanner was the most accurate available for the study. In small calibre-vessels, a change of one pixel tends to correspond to a 20-25% change in luminal dimensions and therefore, the differences in the aortic diameters between the groups may be less significant.

8.4. Haemocompatibility

Signs of blood compatibilty of PLA and stainless steel were assessed by two *in vitro* models with human blood, a steady state model (Study III) and a whole blood perfusion model (Study IV). In all studies, strut thickness of PLA stents represented the diameters of the intravascular stent prototypes designed for experimental application. When compared with control metallic wires (strut diameter 0.1 mm), PLA struts were two to three times thicker (strut diameter 0.2-0.3 mm). Even so, the data from the platelet deposition study showed that at physiological platelet densities, more platelets adhered to stainless steel than to PLA stent struts/strut area. In addition, as evidenced by platelet appearance in representative SEM, platelets on PLA struts showed less activation than on stainless steel struts. Furthermore, heparin coating appeared to strengthen this effect of minimal platelet activation in SEM, but quantitively the number of deposited platelets on heparin-coated stent struts was not diminished. In accordance with this result, Beythien et al. (1999) have shown that heparin coating may not diminish the detectable platelet activation, but it can delay the time until stent thrombosis.

In the assessment of coagulation activation, fibrinogen levels of all materials remained similar to control values (i.e. values for incubation suspensions without a stent strut), as a sign of minimal coagulation activity. In addition, the values of prothrombin fragments F1+2 for PLA resembled those of control values, which tended to be higher for SS struts, but this difference was not significant. The thrombin-time values were significantly prolonged in all assesments for heparin-coated PLA in comparison with stainless steel, which illustrates that the anticoagulant activity of heparin was preserved after the coating process, also under the most thrombogenic conditions (i.e. in the presence of platelets). Both thrombin-antithrombin complex and F1+2 values appeared to be lower for heparin-coated PLA than for SS/PLA surfaces. The difference was statistically significant only for F1+2 at 0.5 µmol/l PPACK/PRP, indicating that in the presence of platelets under limited

anticoagulation (low concentration of PPACK), the heparin-coated PLA strut was an efficient anticoagulant.

The data from the perfusion model (Study IV) showed that under flowing conditions stainless steel stents collected the fewest platelets. As compared to the data obtained in Study III, i.e. PLA presenting at least equal blood compatibility as stainless steel, it can be concluded that many of the material-related positive characteristics of PLA were lost by the stent designs and configurations applied. However, heparin coating with more than 8.3 μg heparin/stent appeared to reduce platelet deposition, although not significantly. When higher releasable heparin doses/metallic stent were applied in another recent study by Matsumoto et al. (2002), platelet deposition appeared to be significantly less on heparin coated surfaces. Whether this finding holds true with PLA must be established by future experiments. Furthermore, according to the current data, in the absence of collagen precoating the plain P(CL/D,L-LA)-coating of PLA stents tended to reduce platelet attachment, but this effect was lost in the presence of collagen, which emphasizes the importance of collagen-dependent activation of platelets. Among all biodegradable stents, the braided PLA stent coated with PCL-PLA-heparin accumulated the fewest platelets. Coagulation activation assessed by F1+2 remained practically unaltered, which accords with earlier findings of local evolution of thrombosis beyond its systemic detection (Mustonen and Lassila 1996, Heemskerk et al. 2000). In accordance with the SEM findings in Study III, the representative studies suggested that the number of platelets deposited does not necessarily indicate enhanced procoagulant activity and fibrin formation: balloon formation on stainless steel and PLA stents, interpreted as local procoagulant activation, was not detected on heparin-coated PLA stents (Figure 4).

Antiplatelet medications targeted to reduce platelet deposition after PTA and stenting have succeeded in diminishing thrombotic events. Heparin and hirudin stent coatings have also been

directed towards this purpose, and experimentally the blood compatibility of both SS and polymeric surfaces appears to be improved (Hårdhammar et al. 1996, Seifert et al. 1995, Alt et al. 2000, Herrmann et al. 1999). Although experimental studies have suggested that controlling the formation of mural thrombi may reduce neointimal overgrowth or restenosis (Matsumoto et al. 2002, Unterberg et al. 1995, Tsuchikane et al. 1999), clinical data have not yet confirmed these findings (Wohrle et al. 2001, the ERASER investigators 1999).

Instead of ionic or covalent bonding of heparin on stents (Table 1), polymer-blended heparin leaching from a stent surface was applied in Studies III and IV. Locally delivered heparin may diminish platelet deposition (Kauhanen et al. 2000, Olsson et al. 2002, Azrin et al. 1994), and in particular, large heparin proteoglycans are highly effective in controlling thrombus growth. To reduce platelet deposition on stent surfaces, Matsumoto et al. (2002) used releasable multilayer-heparin with a dosage/stent approximately four to eight times greater than in Study IV. They found platelet deposition to be diminished, and the effect depended significantly on the heparin dose. The earlier, somewhat disappointing clinical results with ionically or covalently immobilised heparin may partly result from the dual role of immobilised heparin – it may act in initiating the intrinsic pathway of coagulation, despite the antithrombin enhancing activity (Blezer et al. 1998). Moreover, binding competition of antithrombin III with other heparin-binding proteins may diminish the anticoagulant activity of surface-immobilised heparin in plasma, and plasma proteins adsorbing to heparinised surfaces may also inhibit heparin activity (Van Delden 1996). In accordance with earlier data, a recent study demonstrated how blood compatibility can be improved by gradually increasing the concentration of covalently immobilised heparin on a biomaterial surface (Andersson et al. 2003).

8.5. Stent configuration and coating

Stent design affects both platelet activation and neointimal proliferation (Gurbel et al. 2002, Kastrati et al. 2000b, Rogers and Edelman 1995). Thrombotic occlusion of either polymer-covered or polymeric stents has also been reported in experimental models (Zidar et al. 1994, Lincoff et al. 1997, van der Giessen et al. 1996, Susawa et al. 1993). Flexible stents appear to induce less neointimal thickness than rigid stents (Fontaine et al. 1994), and decreased hoop strength may accelerate neointimal overgrowth (Barth et al. 1996). The current in vitro perfusion study indicates that both stent configuration and coating affect platelet attachment also on biodegradable PLA stents. There are a few essential differences in material characteristics between metallic and polymeric stents, however, which markedly influence fabrication properties (Blindt et al. 1999). Dilation of polymeric stents can not be as easily achieved by forming the material at body temperature as metallic stents, because of their plastic forming properties. After withdrawal of the dilating instrument during metallic stenting, the gained deformation remains. Instead, a polymeric stent of a related design will tend to spring back into the original position or even crack and loose its mechanical properties. The polymeric helical design allows the reduction of the stent diameter by twisting the spiral, and that is why it has been considered one of the most promising configurations for polymeric tubular duct devices (Blindt et al. 1999). In the current investigation, the double helical spiral configuration was applied for the animal experiments. Additionally braided designs, partly self-expandable, partly balloon expandable, were produced for the haemocompatibility studies.

There were fewer deposited platelets on stainless steel stents than on PLA stents under all study conditions ($p < 0.03$). PCL-PLA-heparin-coating reduced platelet deposition on both spiral and braided PLA stents, and among all biodegradable stents the braided PLA stent coated with P(CL/D,L-LA)-heparin accumulated the fewest platelets ($p < 0.02$). Coagulation activation in

circulating blood remained unaltered. In representative SEM, signs of platelet activation on braided heparin-coated PLA stents appeared modest when compared with uncoated braided PLA/SS stents. Interestingly, in the absence of collagen precoating, the plain P(CL/D,L-LA) coating of PLA stents seemed to reduce platelet attachment, but this distinction was lost in the presence of collagen. The heparin-coating on braided stents tended to reduce platelet deposition also after collagen exposure. The heparin-coating applied in the current investigation was not sufficient to augment blood compatibility. It is likely that haemocompatibility of PLA stents will increase when previous findings (Matsumoto et al. 2002, Andersson et al. 2003) are taken into account in future stent designs and coatings.

8.6. Clinical significance

The data gained by the current experiments will be helpful in the future in providing tools to explore the biocompatibility of new polymeric materials, but the applied bioabsorbable stent models are still too far from ideal intravascular stents to be suitable for clinical practice. A delightful result was that PLA was at least as haemocompatible as stainless steel as a stent material, but this equivalence was lost in the perfusion experiments with the applied stent models, presumably due to the technical superiority of stainless steel stents. PLA stent designs were still prototypes, and many improvements, including a delicate percutaneous deployment system and an antithrombogenic/ antirestenotic stent coating, should be developed before human trials can begin. As a result, a copolymeric polyester stent could be created, carrying favourable bioactive substances for intravascular therapy, with a strut area less than in the current series, with excellent tissue and blood compatibility, strength, elasticity, and appropriate disintegration time. Radiolucency of these devices will facilitate the follow-up of target areas by less invasive imaging techniques (MRA, CT), and possible long-term complications of permanent non-resorbable devices could be avoided (Colombo and Karvouni 2000). Consequently, applications of stents may extend further.

Combining all biomedical and technological expertise required, in addition to appropriate financial resources, remains a key issue in this effort of creating the ideal vascular stent.

8.7. Future research

The next goals would be to systematically establish the most suitable biodegradation time of selected stent polymers/copolymers for intravascular use, using a miniswine model, as the tissue response may greatly depend on the speed of disintegration. Secondly, stent designs should be developed further, due to the fact that PLA is not inferior to stainless steel as regards material thrombogenicity. However, the real value of adhering platelets in the post-stenting period is unknown. In other words, it still remains to be established the relevance of the current *in vitro* findings by other models before we can be certain if the number of platelets, or signs of their activation, truly represents blood compatibility *in vivo*. Thirdly, any material exposed intravascularly would be thrombogenic and in the long run, potentially restenotic. Therefore, we have to continue to search for intravascular therapies, which in combination with PTA/stenting, would support the healing process towards reendothelialisation and positive remodelling (Welt and Rogers 2002). Whether this could be achieved by endothelial cell implantation, gene therapy, radiation, or drug delivery still remains to be established.

Finally, bioabsorbable materials have been extensively explored for tissue engineering purposes during the past decades. Furthermore, computerised technology is gradually revolutionising clinical decision making and everyday patient care. With advanced computerised models, it is already possible to mimic human pathological conditions. In the future, it may be possible to plan individual therapy for vascular abnormalities, in which not only lesion morphology but also individual parameters, such as comorbidities, physical condition, vascular resistance, coagulative status etc. can be taken into account. At that point, we could end up in a situation where for a

certain symptomatic morphological finding a selection of therapeutic options would be available, but the choice will be *the best for the individual patient*. At that time, (permanent) stents may even be a legend from history.

9. CONCLUSIONS

On the basis of Studies I-IV the following conclusions can be drawn:

1. Biodegradation of poly-L/D-lactide stents in normal rabbit aortas is quite well tolerated. Tissue reactions decline following an initial mild inflammatory phase after six months, and neointimal proliferation is moderate with preservation of luminal patency. The material is totally replaced by fibrosis by two years after implantation. As tissue reactions evoked by a bioabsorbable material remain a target-spesific issue, this must be taken into account before human experiments are planned.

2. In contrast to stainless steel stents, PLA stents allow measurements of luminal patency by magnetic resonance imaging, both immediately after stenting and during the early phase (up to one year) of biodegradation. The PLA stent itself is translucent in conventional 1.5 T MRI.

3. PLA appears to be at least as haemocompatible as stainless steel as a stent material, but this equivalence may be lost under flowing conditions whenever stent design and configuration remains inferior to stainless steel stents, either due to strut thickness and/or area, or mechanical properties.

4. Releasable heparin-coating seems to reduce platelet adherence to PLA stent surfaces, and this effect may be dependent on heparin-dosage.

5. In *in vitro* human blood perfusion models, coating of the perfusion tubing with type I collagen augments the reactivity of platelets in general and may also affect the ability of the coating to act as a thromboresistant surface. This must therefore be taken into account when interpreting data from previous *in vitro* studies and in planning future experiments.

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